AUSTRIA TABAK

CORESTA SMOKE STUDY GROUP
Task Force: Determination of
Citrate in Cigarette Paper

Feb., 1990
Final Draft

1 - Field of Application

The method is applicable to all kinds of cigarette paper.

2 - Definitions

Citrate in cigarette paper influences the burning speed of the cigarette paper and therefore the puff number of the cigarette. Citrate usually is added to the cigarette paper as trisodium salt, as tripotassium salt or as a mixture of trisodium and tripotassium salts.

3 - References

BERGMEYER, H. U. (Hrsgb.) (1974) Methoden der enzymatischen Analyse. 3. Aufl. Br. 2, pp 1609; 1613 - 1615. Verlag Chemie, Weinheim, BRD.

BOEHRINGER Mannheim. (1989) Methoden der enzymatischen Lebensmittelanalytik. Arbeitsanleitungen zur Analyse. Firmenschrift.

ISO 187 Paper and board: Conditioning of samples.

ISO 287 Paper and board: Determination of moisture content - Oven drying method.

4 - Principle

Citric acide (citrate) is converted to oxaloacetate and acetate in the reaction catalyzed by the enzyme citrat lyase (CL) (1).

(1) Citrate $\xrightarrow{CL}$ oxaloacetate + acetate

./2

AUSTRIA TABAK

In the presence of the enzymes malate dehydrogenase (MDH) and L-lactate dehydrogenase (L-LDH), oxaloacetate and its decarboxylation product pyruvate are reduced to L-malate and L-lactate, respectively, by reduced nicotinamide-adenine dinucleotide (NADH) (2) (3).

(2) Oxaloacetate + NADH + H^+ $\xrightarrow{MDH}$ L-malate + NAD^+

(3) Pyruvate + NADH + H^+ $\xrightarrow{L\text{-}LDH}$ L-lactate + NAD^+

The amount NADH oxidized in reactions (2) and (3) is stoichiometric with the amount of citrate. NADH is determined by means of its absorbance at 340 nm.

./3

AUSTRIA TABAK

5 - Materials and equipment

It is recommended to use test kits for the enzymatic citrate determination. Such test kits are available by various suppliers (e.g. BOEHRINGER, Mannheim).

A test kit contains

- a bottle containing 1,4 g lyophilisate, consisting of:
glycilglycine buffer, pH 7,8; malate dehydrogease, 136 U; L-lactate dyhyrdogenase, 280 U; NADH, 6 mg; stabilizers (bottle 1).

- a bottle with 50 mg lyophilisate citrate lyase, 12 U (bottle 2).

Citric acid monohydrate, p.A.

Distilled water

ELRENMEYER flasks, 250 ml.

Calibrated flasks, 1000 ml, 100 ml.

Filtration funnel, 8 cm diameter.

Folded filters, 125 mm diameter.

Pipettes, 10,0 ml, 5,0 ml, 2,500 ml 1,00 ml, 0,200 ml Micropipette.

UV Spectrophotometer, double beam.

Glass cuvettes, volume 5 ml, 10 mm light path.

Ultrasonic bath.

Analytical balance

6 - Standard solutions

For the calibration of the method standard solutions containing 50, 25 and 12.5 ppm citric acid monohydrate p.A. in distilled water are used.

./4

AUSTRIA TABAK

7 - Procedure

Extreme care should be taken by pipetting sample extracts.

a) Preparation of the reagens solutions (for ten determinations).

- Dissolve the content of the bottle 1 of the test kit in 12 ml of distilled water (solution 1)

- Dissolve the content of the bottle 2 of the test kit in 0,3 ml of distilled water (solution 2).

Solution 1 is stable for 2 weeks at +4°C or for 4 weeks at -20°C. Solution 1 has to be brought up to 20 - 25°C before use. Solution 2 is stable for 1 week at +4°C or for 4 weeks at -20°C.

The overall activity of the enzyme systems should be 100 +/- 5 %.

b) Sample preparation.

1,000 g of slized cigarette paper (conditioned according ISO 187) is extracted in 100 ml of distilled water in a 250 ml ERLENMEYER flask for 30 minutes by the aid of an ultrasonic bath. The paper extract is filtered through a folded filter. Reaction solutions with distilled water (blank), with the standard solutions for calibration and with the paper extract are prepared in the following manner:

Pipette into cuvettes	blank	sample and standard
solution 1	1,00 ml	1,00 ml
dist. water	2,00 ml	1,80 ml
paper extract or standard solution	-	0,20ml
mix, read absorbance of the solutions (A_1) after approximatelly 5 minutes, and start the reaction by addition of		
solution 2	0,02 ml	0,02 ml
mix; on completion of the reaction (approximatelly 5 minutes), read the absorbance of the solutions (A_2).		

./5

PM3000984693

Parameters of the UV spectrophotometer:

Wavelength:	340 nm
Cuvettes:	Glass, 5 ml volume, 10 mm light path.
Temperature:	20 - 25 °C
Final volume:	3,02 ml

Read against air (without a cuvette in the light path) or against distilled water.

Determine the absorbance differences ($A_1 - A_2$) for both blank and sample. Substract the absorbance difference of the blank from the absorbance difference of the sample.

$$A = \delta A_{sample} - \delta A_{blank}$$

Occasionally a negative value with $(A_1 - A_2)_{blank}$ is obtained. This value is then to be added to $(A_1 - A_2)_{sample}$ according to the calculation formula.

The absorbance differences measured should as a rule be at least 0,100 absorbance units to achieve sufficently accurate results. If the absorbance difference of the sample (δA_{sample}) is higher than 0,850 (measured at 340 nm), the concentration of citric acid in the sample solution is too high. The sample solution is to be diluted to get a citric acid concentration in the cuvette below of 80 µg.

The absorbance difference is recommended to be between 0,2 to 0,4.

8 - Calculations

According to the general equation for calculating the concentration:

$$C = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times \delta A \text{ [g/l]}, \text{ where}$$

V = final volume [ml]
v = sample volume [ml]
MW = molecular weight of the substance to be assayed [g/mol]
d = light path [cm]
ϵ = absorption coefficient of NADH at 340 nm = 6,3 [l x mol^{-1} x cm^{-1}]

./6

It follows for citric acid (calculated as the anhydrous acid):

$$C = \frac{3{,}02 \times 192{,}1}{6{,}3 \times 0{,}2 \times 1000} \times \delta A = \frac{2{,}90}{6{,}3} \times \delta A$$

C = [g citric acid/l sample solution]

It follows for citric acid (calculated as citric acid monohydrate):

$$C = \frac{3{,}02 \times 210{,}1}{6{,}3 \times 0{,}2 \times 1000} \times \delta A = \frac{2{,}90}{6{,}3} \times \delta A$$

C = [g citric acid monohydrate/l sample solution]

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor F.

If 1,000 g paper is extracted with 100 ml of distilled water, the reading "c" corresponds directly to % citric acid monohydrate or citric acid in the paper at equilibrium humidity.

9 - Specifity and Quality of the method.

The method is specific for citric acid. In collaborative tests, carried out by the CORESTA Smoke Study Group Task Force: "Anayltical methods for Cigarette Papers", a standard deviation of 0,021 % and a coefficient of variation of 5,8 % (0,37 % citric acid monohydrate in the cigarette paper) and a standard deviation of 0,105 % and a coefficient of variation of 4,0 % (2,57 % citric acid monohydrate in the cigarette paper), respectively, was determined.

10 - Analytical Report

The analytical report must contain:

- Brand name of the paper
- Name of the manufacturer or supplier of the paper
- Details of the sampling procedure
- Details of conditioning
- Date of test
- Room temperature and relative humidity in the test room
- humidity of the paper (ISO 287)
- % of citric acid monohydrate in the paper at equilibrium humidity

./7

When paper samples are taken from cigarettes the results might be influenced by external parameters (e.g. additives of the tobacco blend).